

**Pollination ecology of the newly described narrow endemic *Ipomopsis ramosa* (Polemoniaceae) in southwestern Colorado**

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**ABSTRACT**

*Ipomopsis ramosa* is a recently discovered species endemic to a small area of the western San Juan Mountains in southwestern Colorado. The species is closely related to the widespread *I. aggregata* with which it grows sympatrically and appears to be the result of a chance hybrid event between this widespread congener and another species. To understand how reproductive biology may be contributing to the persistence and segregation of *I. ramosa* we completed a series of field investigations to examine the breeding system and pollination dynamics. Our results show that *I. ramosa* is a strictly outcrossing species serviced by two generalist pollinators, *Papilio rutulus* which visits the flowers during its migration early in the season and *Hyles lineata* which serves as the principal pollinator visiting the flowers throughout the flowering season. Floral visitation between both *I. ramosa* and *I. aggregata* was observed; however differences in floral morphology appear to minimize the potential for successful cross-pollination. Published on-line [www.phytologia.org](http://www.phytologia.org) *Phytologia* 99(2): 118-125 (May 9, 2017). ISSN 030319430.

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*Ipomopsis ramosa* Schneider & Bregar is a recently described herbaceous biennial endemic to three small side canyons of the Dolores River in Montezuma and Dolores Counties at the western end of San Juan Mountains in southwestern Colorado (Schneider and Bregar, 2011). The species is restricted to south-facing slopes on red sandstones, siltstones, and shale soils of the Permian age Cutler formation and shows an extent of occurrence of 38.9 km<sup>2</sup> and an area of occupancy of 28 km<sup>2</sup> (Bachman et al., 2011). A recent survey (McCauley, 2016) estimates the total occurrence area to consist of approximately 7000 individuals with all populations consisting of a variety of age classes. Across this range the species grows sympatrically with the widespread *Ipomopsis aggregata* (Pursh) V.E. Grant and molecular phylogenetic analysis indicates that *I. ramosa* is likely derived from hybridization of this widespread congener via long-distance pollen dispersal with an as yet unidentified species of *Ipomopsis* (McCauley et al., 2016). Phylogenetic analysis of nuclear ITS sequence data indicates that two rare species *I. polyantha* (Rydberg) Grant and *I. sancti-spiritis* Wilken & Fletcher are the closest relatives to *I. ramosa*. The rarity of these two taxa suggests they may not be the direct progenitor. One species, the widespread *I. arizonica* (Greene) Wherry could be seen as a potential pollen donor due to its similar floral morphology including short styles and included anthers; however further analysis is needed to verify this assumption. Hybridization within the *I. aggregata* complex is common (Grant and Wilken, 1986) and this pattern of inter-specific hybridization via long-distance pollen dispersal has likely given rise to multiple forms inhabiting small areas across the range of the species (Grant and Wilken, 1988).

The small range, potentially recent hybrid origin, and overlapping distribution with *I. aggregata* suggested that further investigation of the breeding system was important for understanding the species' biology and may give insights to its rarity. Differences in floral morphology between the two species suggest different pollinator preferences which may be serving to keep the species distinct (Aldridge and Campbell, 2007; Kishore et al., 2012) and that a pollinator shift may be involved in maintaining segregation of this hybrid derivative (Van der Neit et al., 2014). *Ipomopsis ramosa* has salmon-colored to

white flowers with inserted stamens while *I. aggregata* flowers are red and generally express exerted stamens (Schneider and Bregar, 2011) although some higher elevation populations in the region may express inserted stamens. Another potential for maintaining species segregation is the possibility of selfing. While complete autogamy is not reported for the genus *Ipomopsis* and selfing is rare (Grant and Grant, 1965; Waser and Price, 1991) the close relative *I. polyantha* has been shown to set seed via selfing (Collins, 1995) and thus there is potential for expression of that trait in *I. ramosa*. Low seed set due to the hybrid derivative nature of the species (Grant, 1966) or low levels of pollination due to pollinator rarity or inconsistent visitation due to the restricted range (Wilcock and Neiland, 2002) may additionally be limiting dispersal ability.

To evaluate pollination and breeding system ecology of *I. ramosa* we addressed the following specific questions:

- 1) What is the identity of the principal pollinator(s)?
- 2) What is the potential contribution of both selfing and outcrossing for fruit production?
- 3) What is the pattern of pollen movement between *I. ramosa* and the sympatric *I. aggregata*?
- 4) What are the structural differences between flowers of *I. ramosa* and *I. aggregata* which may be serving to limit gene flow?

## MATERIALS AND METHODS

We performed field studies during the summer of 2014 in two side canyons of the Dolores River ca. 38 km northeast of the town of Dolores, Colorado. These two canyons, Roaring Forks Canyon (37°35'13"N, 108°05'44"W) and Wildcat Canyon (37°37'31"N, 108°04'18"W) represent the two largest occurrence areas for *I. ramosa*. Prior observations (Breedon, unpublished data) indicated that flowering begins in late May, generally peaking by mid-July and finishing by mid-August. Field trials and observations were thus timed to span this flowering period.

### *Breeding system*

We tested the potential for selfing vs. outcrossing using pollinator exclusion (Kerns and Inouye, 1993). Thirty individuals in Roaring Forks Canyon were chosen in June prior to anthesis and 15 of these were covered with a mesh of 1 mm diameter. The remaining 15 individuals were left uncovered. From June through August we counted the number of flowers on each individual twice per month. Fruit production was also monitored until maturity. At maturity we collected all fruits intact. Counts of flowers, fruits, and seeds were made for each group and compared using a t-test in SigmaPlot ver. 13.

### *Pollinator identification*

A collective 32 hours were spent in Roaring Forks Canyon spread over different times of the day throughout the flowering period observing and capturing possible pollinators. We captured floral visitors with nets and placed them in a collection jar with ethyl acetate for later identification. To verify insects as pollen carriers, we removed pollen from the proboscis of collected insects, observed it with light microscopy at 1000x, and compared it visually with pollen grains from *I. ramosa*. Due to the similarity in pollen morphology between *I. ramosa* and *I. aggregata* it was not possible to differentiate between the two species.

### *Pollen movement*

To investigate patterns of pollen movement we used fluorescent dyes as a pollen analogue (Waser and Price, 1982; Fenster et al., 1996; Van Rossum et al., 2010). Four trials were conducted, three in Roaring Forks Canyon and one in Wildcat Canyon in July and August 2014. Slight differences in species abundance were observed between the two sites with *I. ramosa* being the dominant of the two species in Roaring Forks Canyon and *I. aggregata* being dominant in Wildcat Canyon. Fluorescent dye powder (Radiant Color Co., Richmond, CA) was applied to all flowers of 1-2 individuals of each species at peak

anthesis growing at central locations within the study area. Each species was painted a different color to assess movement of pollen of each and pigment color was alternated between species at each trial to account for potential pollinator color preference. Dye was applied with a small soft paintbrush to the opening of the corolla and moved downward into the floral tube by gently tapping the flower. Following application of pigment the sites were vacated for 24 hours. Identification of pollen movement was verified by noting the occurrence of dye particles on any flower of an individual plant within a roughly circular area around the dye-source individuals. At Roaring Forks Canyon these circular plots were roughly 150 m<sup>2</sup>. Due to a wider spacing of individuals at Wildcat Canyon the plot was larger at approximately 700 m<sup>2</sup>. During low light hours each flower within these circular plots was inspected for florescent pigment using a handheld blacklight. Following inspection, the dye-source individuals were removed from the study site and the area given several weeks to purge any remaining pigment before another trial was conducted.

#### *Floral morphology*

Twenty flowers of both *I. ramosa* and *I. aggregata* were collected at various locations and elevations throughout Roaring Forks Canyon. These flowers were pressed and dried and measurements taken on the dried flowers of the floral tube, pistil and stamen length. We compared measurements for differences using a t-test in SigmaPlot ver. 13.

## RESULTS

#### *Breeding system*

The number of flowers produced by both selfing and outcrossing groups was found to not be significantly different (Figure 1). Fruit production however was significantly different between the two groups ( $p < 0.0001$ ) (Figure 1). Seed set was estimated from fruits in outcrossed plants and found to be 3.1.

#### *Pollinator identification*

Two pollinators were observed, captured, and confirmed to be carrying pollen of *I. ramosa*. *Papilio rutulus*, the Western Tiger swallowtail, a generalist butterfly in the family Papilionidae was observed on *I. ramosa* in the beginning of June and not noted at any later time (Figure 2). *Hyles lineata*, the Hummingbird Moth, a generalist species of moth in the family Sphingidae was observed visiting *I. ramosa* from mid-June until the middle of August when flowering ceased. Hummingbirds (*Selasphorus spp.*), a common pollinator of *I. aggregata*, were observed in the study area but were not observed visiting individuals of *Ipomopsis*.

#### *Pollen movement*

Pollen movement was principally intraspecific although interspecific pollen exchange was observed in all four trials (Figure 3). In each of the three trials conducted in Roaring Forks Canyon, interspecific pigment transfer moved from *I. aggregata* to *I. ramosa*. At Wildcat Canyon, the opposite was true; pigment was transferred from *I. ramosa* to *I. aggregata*.

#### *Floral morphology*

Flowers of *I. ramosa* were found to be significantly shorter than those of *I. aggregata* in respect to floral tube length, stamen length and pistil length ( $p < 0.01$ ) (Figure 4). The most evident difference was stamen position. Stamens were found to be included within the floral tube in *I. ramosa* while they were exerted in *I. aggregata*.

## DISCUSSION

Our results indicate that seed set in *I. ramosa* requires pollinator visitation and does not exhibit the potential for selfing as seen in its close relative *I. polyantha* (Collins, 1995). The low level of seed set seen in the pollinator exclusion experiment is likely the result of rare chance pollination due to a loose fitting bag covering an inflorescence. This indicates that while *I. ramosa* is likely of hybrid origin this did not lead to the evolution of self-fertility. While the action of a pollinator is required for seed set our experiment did not differentiate between pollen exchanges among flowers of the same individual and thus the potential for geitonogamy cannot be fully ruled out. Further examination via manipulation of floral structures would be necessary to evaluate this question.

The two floral visitors observed on *I. ramosa* were both generalist, polylectic species which have been commonly reported visiting species of Polemoniaceae, including *Ipomopsis* (Grant and Grant, 1965). *Papilio rutulus*, only observed for a short period of time in June, is a migratory species making significant movements at various times during the summer months (Scott, 1986). While not likely serving as the principal pollinator due to its short time in the population this species is likely important as it could serve as a vector for long-distance pollen movement connecting this endemic species with others across a wide area. This potential for long-distance pollen movement and subsequent cross-pollination has been invoked as a plausible mechanism for the recent origin of this species (McCauley et al., 2016).

*Hyles lineata*, which we observed visiting the population for an extended period of time during most of the flowering period is a local resident species. This species likely serves as the principal pollinator of *I. ramosa*. Based on floral form and general pollination syndrome this should serve to isolate *I. ramosa* from *I. aggregata* and provide evidence for a pollinator shift in the evolution of *I. ramosa*. *Ipomopsis aggregata* with its red tubular flowers is principally pollinated by hummingbirds (Grant and Grant, 1965) although various Sphingidae species, including *H. lineata* are often seen associating with and occasionally pollinating it (Juenger and Bergelson, 1997). While *H. lineata* will more often associate with lighter white to pink flowers the species has been shown to act as a generalist, particularly in high elevation areas. This is mostly driven by the coldness of the areas limiting *H. lineata* activity at night when light floral colors are most distinct to the pollinator (Aldridge and Campbell, 2007). Thus while *I. aggregata* is likely being pollinated by hummingbirds, and we did observe hummingbird (*Selasphorus spp.*) activity in the area of Roaring Forks Canyon, floral visitation is being made by *H. lineata* providing the possibility for pollen exchange.

The examination of pollen movement between *I. ramosa* and sympatric *I. aggregata* indicated the occurrence of pollen exchange between the two species. All trials indicated that interspecific movement while less common than intraspecific movement, did occur. This greater frequency of intraspecific pollen movement suggests that there may be pollinator preference and division based on the servicing pollinator. Interspecific pollen movement was likely facilitated by the generalist behavior of *H. lineata*. Floral preference by *H. lineata* cannot be evaluated here as pollinator observations were not made on *I. aggregata* but assuming patterns seen by other Sphingidae *I. aggregata* would be of secondary preference to the lighter colored *I. ramosa*. Assuming however that *H. lineata* may not show any preference between the two species of *Ipomopsis* the same pattern of pollen flow could be explained by the pattern of clumping that the species exhibit in the field. Nearest-neighbor visitation may be the principal explanation for the observed pattern. Further pollinator observations are needed to differentiate these two driving forces behind the observed pattern of interspecific pollen flow.

Morphological analysis indicates that mechanical isolation in the form of floral structure is an important mechanism for isolation of *I. ramosa*. Evidence points to some floral traits in *Ipomopsis* being under relatively simple genetic control and thus these differences could have evolved relatively rapidly (Wolf and Soltis, 1992). With the potential for pollen flow between *I. ramosa* and *I. aggregata*,

differences in morphology, particularly the placement of stamens and stigmas, may be preventing cross-pollination. The differences in stamen and stigma insertion however suggest a potential for asymmetric pollen flow with *I. ramosa* having inserted stamens being able to fertilize *I. aggregata*, but *I. aggregata* not being able to fertilize *I. ramosa*. Such asymmetrical differences in pollen movement have been previously observed in other closely related and hybridizing members of *Ipomopsis* (Wolf et al., 2001) and play important roles in pollen transfer. If this apparent pattern stands up it would mean that *I. ramosa* can be maintained as a genetically isolated population and may be able to spread via introgression with *I. aggregata*. Further work to investigate pollen fertility following exchange in both species and the patterns of gene flow in these populations would be necessary to fully determine the level of interspecific gene flow and potential for introgressive hybridization.

Our work shows that the narrow endemic *I. ramosa* is an outcrossing-dependent species serviced by a generalist pollinator and maintained via distinct patterns of floral form. The rarity of *I. ramosa* is thus likely a result of a chance interspecific hybridization with *I. aggregata* which gave rise to a species with different floral form. This difference in form gave rise to a partial pollinator shift utilizing a generalist pollinator with cross-pollination controlled by mechanical differences in flower structure with sympatric *I. aggregata*. Given the current stability of the known populations and their reliance upon a common generalist pollinator it does not appear that this rare species faces any threats associated with reproduction and breeding system.

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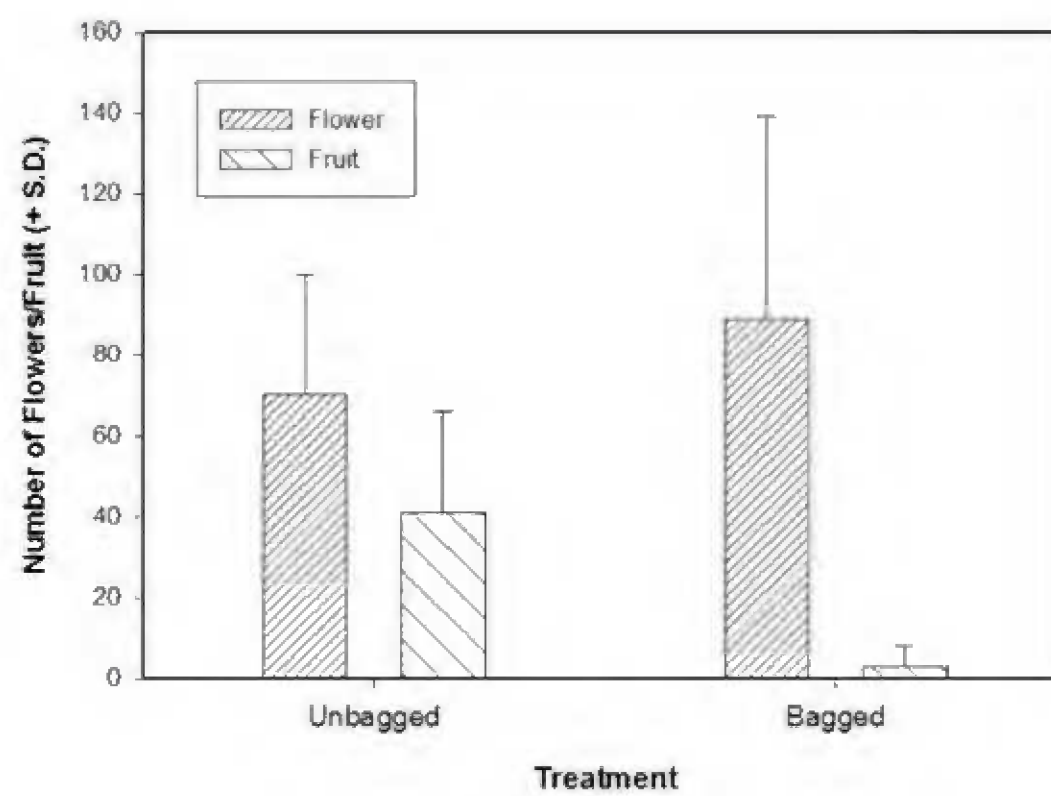


Figure 1. Average (+ S.D.) number of flowers and fruits produced in outcrossing and pollinator exclusion treatments in *Ipomopsis ramosa*.



Figure 2. *Papilio rutulus* visiting flowers of *Ipomopsis ramosa* in Roaring Forks Canyon.

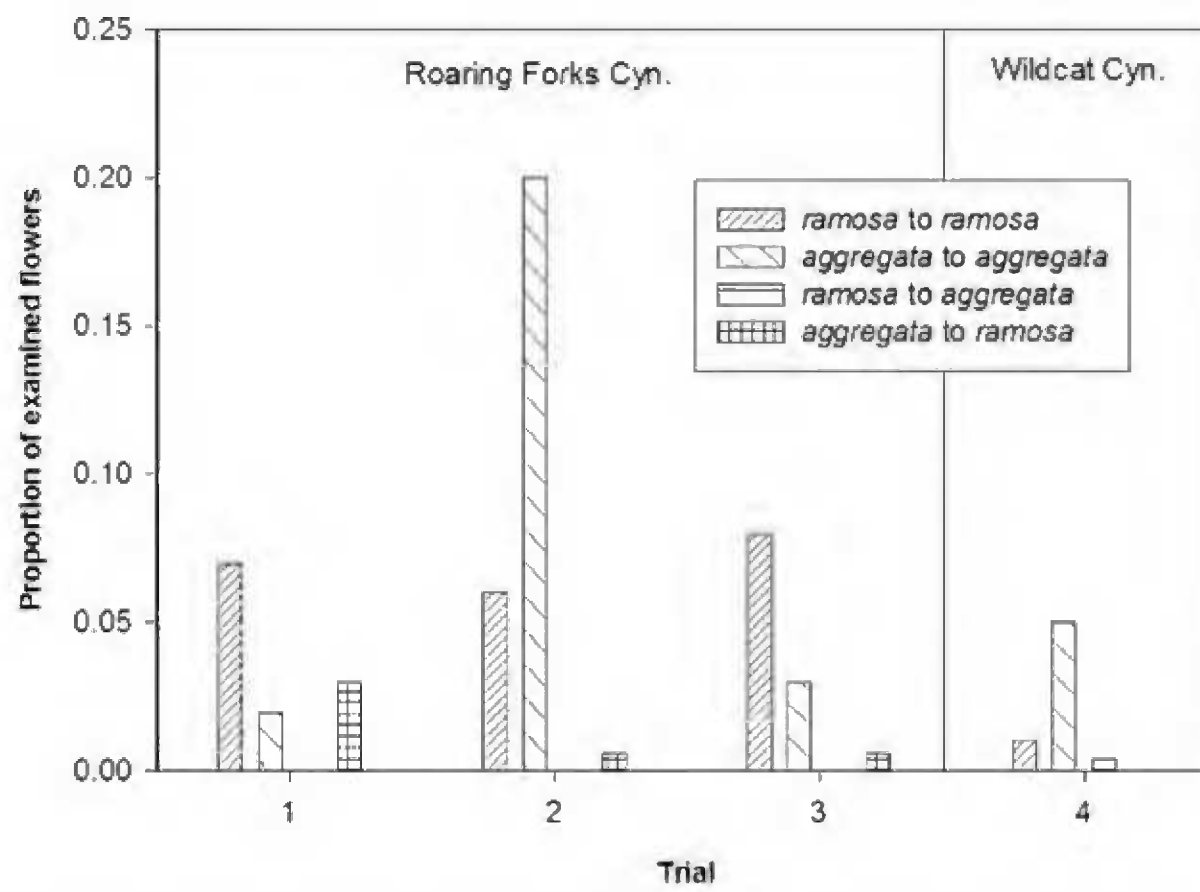


Figure 3. Proportion of examined flowers expressing transported dye particles in four trials across two areas of *Ipomopsis ramosa* occurrence.

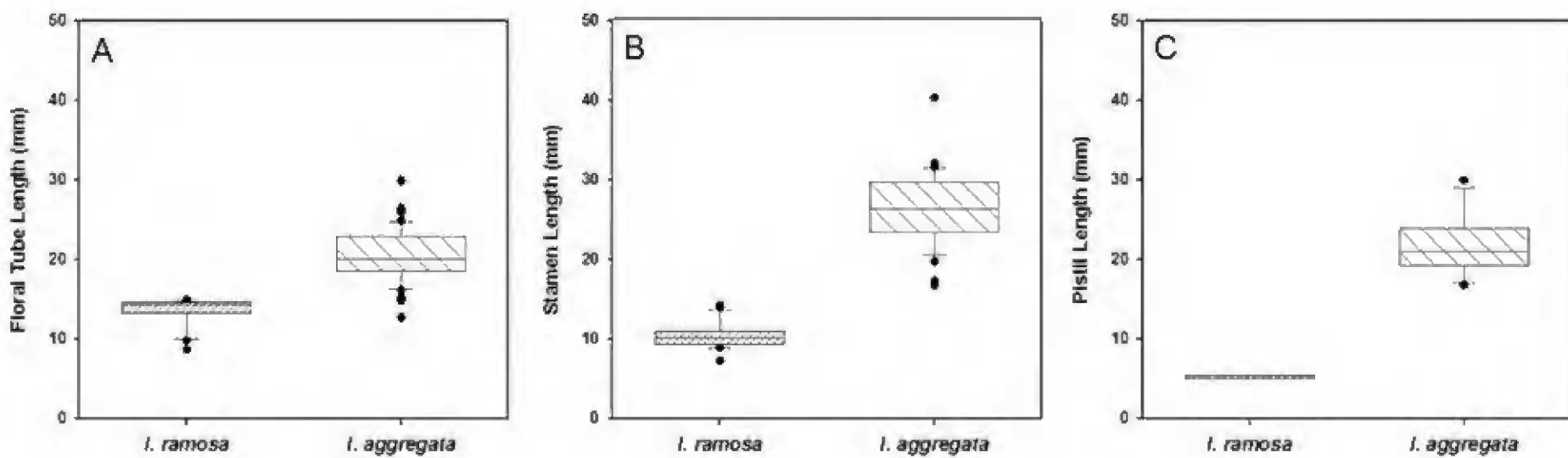


Figure 4. Comparison of floral morphology between *Ipomopsis ramosa* and *Ipomopsis aggregata* growing sympatrically in Roaring Forks Canyon. A. Floral tube length. B. Stamen length. C. Pistil length.